Prostaglandin $F_{2\alpha}$ upregulates *Slit/Robo* expression in mouse corpus luteum during luteolysis

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Abstract

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a key factor in the triggering of the regression of the corpus luteum (CL). Furthermore, it has been reported that *Slit/Robo* signaling is involved in the regulation of luteolysis. However, the interactions between $PGF_{2\alpha}$ and *Slit/Robo* in the progression of luteolysis remain to be established. This study was designed to determine whether luteolysis is regulated by the interactions of $PGF_{2\alpha}$ and *Slit/Robo* in the mouse CL. Real-time PCR and immunohistochemistry results showed that *Slit2* and its receptor *Robo1* are highly and specifically co-expressed in the mouse CL. Functional studies showed that *Slit/Robo* participates in mouse luteolysis by enhancing cell apoptosis and upregulating caspase3 expression. Both *in vitro* and *in vivo* studies showed that $PGF_{2\alpha}$ significantly increases the expression of *Slit2* and *Robo1* during luteolysis through protein kinase C-dependent ERK1/2 and P38 MAPK signaling pathways, whereas an inhibitor of *Slit/Robo* signaling significantly decreases the stimulating effect of $PGF_{2\alpha}$ on luteolysis. These findings indicate that *Slit/Robo* signaling plays important roles in $PGF_{2\alpha}$ -induced luteolysis by mediating the $PGF_{2\alpha}$ signaling pathway in the CL.

Key Words

- ▶ *Slit/Robo*
- ▶ prostaglandin $F_{2\alpha}$
- ▶ luteolysis
- ▶ mouse

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Introduction

The corpus luteum (CL) is a transient endocrine gland that develops when the ovulated follicles are transformed through a terminal differentiation process termed luteinization (Stocco *et al.* 2007). The main functions of the CL are the regulation of the estrous cycle and the maintenance of pregnancy. If pregnancy does not occur, the CL regresses through a process termed luteolysis. In rodents, CL regression includes the functional and structural phases. The functional phase is associated with a marked decrease in progesterone production, which is followed by the structural phase in which the luteal cells die through programmed cell death (Hernandez *et al.* 2011).

CL structural regression is characterized by a reduction in both size and weight. The CL eventually becomes a cluster of cells termed the corpus albicans. The molecular mechanisms of CL structural regression are not yet clear, but the major events include the apoptosis of luteal and vascular cells (Stocco *et al.* 2007). It is well established that the apoptosis of luteal cells comprises two distinct signaling pathways: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway (Yadav *et al.* 2005, Stocco *et al.* 2007). The intrinsic pathway is activated by apoptotic stimuli, such as drugs, radiations, and cytokines (Adams & Cory 1998), and the extrinsic pathway is activated by the interaction between the death

ligands and death receptors (Kuranaga *et al.* 1999, Roughton *et al.* 1999, Sartorius *et al.* 2001). Several signals have been implicated in the induction of the apoptosis of luteal cells, including prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$), progesterone, prolactin, and Fas ligand (FasL; Bowen *et al.* 1996, Roughton *et al.* 1999, Gaytan *et al.* 2000, Kuranaga *et al.* 2000, Taniguchi *et al.* 2002, Carambula *et al.* 2003, Yadav *et al.* 2005). It has been reported that PGF $_{2\alpha}$ interacts with its G-protein-coupled receptor; this interaction increases the ratio of *Bax* to *Bcl2*, thus elevating the protein levels and activity of caspase9 and caspase3 (Yadav *et al.* 2005).

The *Slit/Robo* family comprises four transmembrane Robo (1–4) receptors that interact with their *Slit* (1–3) ligands. The *Slit/Robo* family regulates cell fate, including migration, death, angiogenesis, and organogenesis (Wu *et al.* 2001, Park *et al.* 2003, Dickinson *et al.* 2004, Hinck 2004, Koch *et al.* 2011). It has been documented that *Slit/Robo* suppresses the development of cancers by inhibiting cell migration and promoting apoptosis (Dickinson *et al.* 2008), and both *Slits* and *Robos* are inactivated in several tumors including cervical, prostatic, and ovarian tumors (Latil *et al.* 2003, Singh *et al.* 2007, Dai *et al.* 2011).

Under physiological conditions, *Slit/Robo* is expressed in both fetal and adult ovaries and participates in the regulation of follicle formation, oocyte survival (Dickinson *et al.* 2010), menstrual cycle (Duncan *et al.* 2010), and luteolysis by promoting the apoptosis of luteal cells (Dickinson *et al.* 2008). However, the factors affecting the expressions of *Slit/Robo* and their relationship with luteolysis require further investigation. Since PGF $_{2\alpha}$ is a key factor in the induction of CL regression, we hypothesized that *Slit/Robo* might interact with PGF $_{2\alpha}$ to affect CL regression. Our results indicate that PGF $_{2\alpha}$ stimulates the expression of *Slit/Robo* in both cultured isolated luteal cells and CL tissues and the effect of PGF $_{2\alpha}$ on the apoptosis of luteal cells is mediated by the *Slit/Robo* interaction.

Materials and methods

Reagents

Rabbit IgG anti-Slit2 polyclonal antibodies and mouse IgM anti-Robo1 MABs were purchased from Abcam, Inc. (Cambridge, MA, USA) and Developmental Studies Hybridoma Bank (DSHB, Iowa City, IO, USA) respectively. Biotin-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Biotin-conjugated goat anti-mouse IgM, HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgM, and goat anti-mouse IgG were obtained

from ZSGB-Bio, Inc. (Beijing, China). Fluorescein isothiocyanate (FITC) and tetraethyl rhodamine isothiocyanate-conjugated streptavidin were obtained from Southern Biotech (Birmingham, AL, USA). PGF $_{2\alpha}$, collagenase II, PKA inhibitor (H89), PKC inhibitor (CH), and JNK inhibitor (SP600125) were obtained from Sigma–Aldrich. ERK inhibitor (PD98059), P38 inhibitor (SB203580), and Moloney Murine Leukemia Virus (M-MLV) were purchased from Promega. DMEM/F12 and fetal bovine serum (FBS) were obtained from Gibco, and 0.25% pancreatin was obtained from Amresco, Inc. (Solon, OH, USA). Percoll was purchased from GE Healthcare Life Sciences (Castle Hill, NSW, Australia). Recombinant rat ROBO1/Fc chimera was obtained from R&D Systems (Minneapolis, MN, USA). *In situ* apoptosis analysis kit was purchased from Roche Diagnostics. RIPA lysis buffer and BCA assay reagent were purchased from Biotech Corporation (Beijing, China). PVDF membranes were obtained from Bio-Rad Laboratories. SuperSignal West Pico kit was purchased from Thermo Scientific (Rockford, IL, USA). All the other reagents were purchased from Takara (Tokyo, Japan) or TianGen Biotech Co., Ltd (Beijing, China).

Animals

Twenty-one-day-old female Kunming white mice were purchased from the Animal Institute of the Chinese Medical Academy (Beijing, China) and were raised under standard conditions of temperature ($25 \pm 1^\circ\text{C}$) and light (12 h light:12 h darkness cycle). These mice were i.p. injected with 10 IU pregnant mare serum gonadotropin (PMSG) to stimulate follicle development, which was followed 48 h later by an injection of 10 IU human chorionic gonadotropin (hCG) to promote ovulation and to obtain luteinized ovaries. These mice were then mated with castrated male mice. Day 0 was taken as the day of hCG injection. According to previous studies (Olofsson & Selstam 1988, Hasumoto *et al.* 1997), the ovaries were categorized as early (D0–D5), mid- (D6–D10), and late (D11–D15) luteal phase, and the PGF $_{2\alpha}$ content and DNA fragmentation in the CL was increased at D11 of this animal model. All animal procedures were approved by the Chinese Association for Laboratory Animal Sciences.

Mouse CL collection

Ovary collection in the mice at different luteal phases PMSG–hCG-synchronized ovulation and luteinization were induced and the ovaries were collected from

the mice at the early, mid-, and late luteal stages ($n \geq 3$ at each stage).

Ovary collection in the mice after cloprostenol injection On D6 after hCG injection, the mice were i.p. injected with cloprostenol, a synthetic analog of PGF_{2 α} , and the ovaries were collected at 0, 4, 12, and 18 h after cloprostenol injection ($n \geq 3$ mice/time point).

The ovaries were collected and washed in PBS. Under sterile conditions, CL tissues were enucleated from the ovaries under a microscope with the aid of fine forceps. The CL tissues were stored at -80°C until analysis.

CL tissue isolation and luteal cell culture

CL tissues obtained from the mice on D6 after hCG injection were transferred into a centrifuge tube containing 0.1% collagenase II as described previously (Thordarson *et al.* 1997). Enzymatic digestion was carried out in a shaking bath (130 r.p.m./min) at 37°C for 1 h. In order to obtain individual cells, tissue pieces were further dispersed by withdrawing and expelling at the end of the digestion. The supernatant, containing individual cells, was removed and transferred into another centrifuge tube. Undissociated clumps were further incubated in 0.25% pancreatin in a shaking bath at 37°C for 10 min. After the termination of digestion, the cell suspension was filtered and layered onto a 2 ml cushion of 44% percoll in a centrifuge tube and centrifuged at $400g$ for 30 min. The luteal cells that banded at the interface between the percoll and the medium were harvested, washed, and resuspended in DMEM/F12 medium containing 10% FBS. The cells were then counted and viability was assessed using trypan blue exclusion test; the viability varied from 85 to 95% in cell preparations used for further study. For the assay, the cells were plated (1.0×10^5 cells/well) onto six-well plates for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . The cells were then serum starved for an additional 24 h and then incubated using different treatments. Luteal cells from 20 to 25 mice were collected for each culture.

Immunohistochemistry

Frozen sections of the ovaries ($7\text{ }\mu\text{m}$) were fixed with cold methanol for 10 min, subjected to microwave antigen retrieval in 0.01 M citric acid (pH 6.0) for 15 min, and left to cool at room temperature (RT). All sections were then treated with 10% normal goat serum at RT for 1 h and incubated with rabbit IgG anti-Slit2 (1:100) antibodies at

4°C overnight. The sections were then incubated with biotin-conjugated goat anti-rabbit IgG (1:400) at RT for 3 h. Subsequently, the slides were incubated with FITC-conjugated streptavidin (1:25) for 3 h at RT. For dual staining, the slides were further incubated with mouse IgM anti-Robo1 (1:100) at 4°C overnight and biotin-conjugated goat anti-mouse IgM (1:100), and tetraethyl rhodamine isothiocyanate-conjugated streptavidin (1:25) at RT for 3 h. The sections were counterstained with DAPI (1:1000) to enable cell identification. As negative controls, the sections were processed as described above, except that the primary antibody was replaced with blocking serum containing nonspecific immunoglobulins at the same concentration. The slides were imaged using a fluorescence microscope (Leica Microsystems, Cambridge, UK).

Expression analysis

According to the protocols provided by the manufacturer, total RNA of the CL tissues and primary luteal cells were isolated using the TRIzol reagent, purified by DNase I, and quantified by spectrophotometry. Purified total RNA ($1\text{ }\mu\text{g}$) was used as a template for cDNA synthesis using the M-MLV, according to the manufacturer's instructions. All reverse transcriptase reactions included no-template controls and minus controls. In all, $2\text{ }\mu\text{l}$ of each cDNA were used for amplification reactions. Primers were designed using Primer 5.0, and they are described in Table 1. The PCR was continued for 35 cycles after an initial denaturation step at 94°C for 10 min. Each PCR cycle consisted of steps carried out at 94°C for 30 s and annealing temperature at 72°C for 30 s, as well as a final extension step for 10 min at 72°C . The PCR products were subsequently size verified by agarose gel electrophoresis with ethidium bromide and were observed and photographed under u.v. light. The relative intensity of each blot was assessed and analyzed using the AlphaImager 2200 Software package (Alpha Innotech Corp., San Leandro, CA, USA). For quantification, *Slit2/Robo1* mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which was used as the housekeeping gene.

Real-time quantitative PCR

RNA was extracted and reverse transcribed as described above. Real-time PCR was performed using a standard Takara SYBR Premix Ex Taq protocol on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Primers used were the same as those used for the expression analysis, and they are described in Table 1. The reaction mixtures were

Table 1 Primers used in the expression analysis of candidate genes. Each of the genes investigated, primer sequences, specific annealing temperature used to amplify each product, and product size are given

Gene	Primer sequence (5'–3')	AT (°C)	Product size (bp)
<i>Slit1</i>	F: GGGCCATGTCCGTGTTAG R: TGTAAGTCTTCCAAAGTTGT	60	179
<i>Slit2</i>	F: ATTAGTGAAGCGGTGGGTAC R: CCTTGGGAAGTGTGTGAA	60	159
<i>Slit3</i>	F: GGACAATGGCATCCTTCTTT R: CCCACTGCTGGTTGCTTCT	60	246
<i>Robo1</i>	F: GCATAGGTATCAGGCTTGACC R: TTCCTTAGAAGTGCACATCC	60	196
<i>Robo2</i>	F: AGTCACGGCAGACCCAA R: TTCATAGCCCTGTAGTCTCCTA	60	167
<i>Robo3</i>	F: CACCCAGATGCTGCACTTC R: GGCTCCGGCTTCGACTT	60	196
<i>Robo4</i>	F: TAAAGGAGAAAGGTCGTGGATG R: GAGTGGCGGTAGAATGAGAATAG	60	140
<i>Bax</i>	F: TTTATCCAGGATCGAGCAGG R: GCAAAGTAGAAGAGGGCAACCAC	56	264
<i>Bcl2</i>	F: CTACCGTCTGACTTCGCA R: TACCCAGCCTCCGTTATCC	56	268
<i>Caspase9</i>	F: CGGAATCACCAATCATTACAT R: AGAAACGCCACAACCTGC	53	346
<i>Caspase3</i>	F: AGAGGAATGATTGGGGGTG R: TTGCTAGGCAGTGGTAGCG	56	133
<i>Gapdh</i>	F: GGTGTCTCTGCGACTTCA R: GGGTGGTCCAGGGTTTCTTA	60	186

F, forward primer; R, reverse primer; AT, annealing temperature.

incubated in 96-well plates at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was assayed in triplicate. The PCR products were confirmed to be *Slits/Robos* by sequencing. The melting curve in the PCR indicated a single product yield using this method, and the relative abundance of the genes was determined using the ABI PRISM 7500 equipped software (Applied Biosystems). The results of real-time PCR products were normalized to their respective control, *Gapdh*, which was used as the housekeeping gene.

Western blotting

To detect SLIT2 and ROBO1 protein levels in the CL by western blotting, CL tissues were first lysed using RIPA lysis buffer. The protein concentration of each group was determined using the BCA assay reagent. Equal amounts of protein (50 µg) were electrophoresed on 8 and 12% SDS-PAGE gel for SLIT2/ROBO1 and GAPDH (internal control) respectively, and the bands were transferred onto PVDF membranes. The membranes were blocked and incubated at 4 °C overnight with rabbit IgG anti-Slit2

(1:500), mouse IgM anti-Robo1 (1:500), or mouse IgG anti-GAPDH (1:40 000) antibodies in TBS. The PVDF membranes were then washed three times for 30 min in TBST (0.1% Tween 20 in TBS) and incubated for 2 h with HRP-conjugated goat anti-rabbit IgG (1:3000), goat anti-mouse IgM (1:3000), or goat anti-mouse IgG (1:3000). After washing for 30 min, the membranes were treated with the SuperSignal West Pico kit substrate at RT for 1–5 min. As negative controls, membranes were processed as described above, except that the primary antibody was replaced with nonimmune bovine serum at the same concentration. The relative intensity of each blot was assessed and analyzed using the AlphaImager 2200 Software package, and the levels of SLIT2/ROBO1 were determined by normalization against the density of GAPDH.

In situ apoptosis analysis

The *in situ* apoptosis of cells was detected using the TUNEL technique. Briefly, cell samples were fixed with a freshly prepared fixation solution (4% PFA) for 1 h at 15–25 °C and then incubated with a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The cells were washed three times in PBS and incubated with the TUNEL reaction mixture containing TdT and fluorescein-dUTP for 1 h at 37 °C in the dark. After washing with PBS, the cells were counterstained with DAPI (1:1000) for 15 min. As negative controls, the fixed and permeabilized cells were further incubated with fluorescein-dUTP solution (without TdT) instead of with the TUNEL reaction mixture. Lastly, the incorporated fluorescein was visualized with a fluorescence microscope.

Statistical analysis

All experiments were independently performed three times with different mice ($n \geq 3$ mice/group) or cell preparations in each experiment. Qualitative data reported are representative results obtained in the replicate experiments and presented as means $\pm$ S.E.M.. Statistical analysis was performed using SPSS 10.0 (SPSS, Inc.). The *t*-test was used to compare the treatment and control samples, and one-way ANOVA was used when more than two groups were compared. When differences were observed using ANOVA, pairwise comparisons were made using the *t*-test. Differences are given as * $P < 0.05$; ** $P < 0.01$; or *** $P < 0.001$. A *P* value < 0.05 was considered to be statistically significant.

Results

Slit2 and *Robo1* are highly expressed in the mouse CL

We initially determined the expression of the *Slit* and *Robo* family members in the mid-luteal-phase CL (mid-CL) using real-time PCR. The results showed that the relative abundance of *Slit2* mRNA was much higher than that of both *Slit1* and *Slit3* mRNAs. Among the *Robo* family, *Robo1* mRNA levels were higher than *Robo1*, *Robo2*, *Robo3* and *Robo4* mRNA levels in the CL (Fig. 1A). We then assessed *Slit2* and *Robo1* mRNA levels at different luteal phases, and the results showed that *Slit2* mRNA levels were significantly higher at the late stage than at the early and mid-luteal stages (Fig. 1B, $P < 0.05$). It is interesting to note that the expression level of *Robo1* was also highest at the late stage corresponding to that of its ligand *Slit2* (Fig. 1B, $P < 0.01$). Using immunohistochemistry (IHC), we then located SLIT2 and ROBO1 in the late-luteal-stage ovaries of mice. The results showed that SLIT2 and ROBO1 were co-localized in the luteal cells of the mouse CL, and almost no positive signal was observed in the follicular and stromal cells (Fig. 1C). These demonstrate that *Slit2* and *Robo1* are highly and specifically expressed in the mouse CL during the late stage and are probably involved in the regulation of luteolysis.

Slit/Robo interaction in the apoptosis of mouse luteal cells

In order to determine whether the *Slit/Robo* interaction is involved in the apoptosis of luteal cells in mice, we blocked *Slit/Robo* signaling in cultured luteal cells with a ligand trap consisting of a recombinant ROBO1/Fc chimera (0.1 $\mu\text{g/ml}$) or an equivalent volume of PBS/0.01% (w/v) BSA (control) for 72 h, and cell apoptosis was measured using an *in situ* cell death detection kit (Fig. 2A). We detected apoptosis in more than 5000 cells per treatment. In the ROBO1/Fc chimera-treated group, there were roughly 38% less apoptotic cells than in the control group (Fig. 2B). In addition, the expression of caspase3 was significantly decreased in the ROBO1/Fc chimera-treated group than in the control group (Fig. 2C, $P < 0.05$). These data confirm that *Slit/Robo* signaling enhances the apoptosis of luteal cells.

PGF $_{2\alpha}$ increases *Slit2/Robo1* expression in cultured luteal cells

Since PGF $_{2\alpha}$ is known to be a key factor in the induction of luteolysis (Pharriss *et al.* 1972), we hypothesized that

PGF $_{2\alpha}$ induces luteolysis by affecting the expression of *Slit/Robo* in the CL. To confirm this presumption, luteal cells were cultured in DMEM/F12 medium with the addition of 0 (control), 0.01, 0.1, and 1 μM PGF $_{2\alpha}$ for 6 h, and the effect of PGF $_{2\alpha}$ on the expression of *Slit2/Robo1* in the luteal cells was measured. The results showed that 0.1 and 1 μM PGF $_{2\alpha}$ significantly increased *Slit2* mRNA levels ($P < 0.05$), but 0.01 μM PGF $_{2\alpha}$ had no obvious effect on the expression of *Slit2*. However, all doses of PGF $_{2\alpha}$ used increased *Robo1* mRNA levels in a dose-dependent manner (Fig. 3A, $P < 0.01$ and $P < 0.001$).

We then examined *Slit2* and *Robo1* mRNA levels after incubating the cells with 1 μM PGF $_{2\alpha}$ for 0, 3, 6, 12, and 24 h. The results showed that there was a gradual increase in *Slit2* and *Robo1* mRNA levels from 0 to 12 h. However, the enhancing effect of 1 μM PGF $_{2\alpha}$ on the expression of *Slit2* and *Robo1* decreased after the cells were cultured for 24 h (Fig. 3B). These data reveal that PGF $_{2\alpha}$ upregulates the expression of *Slit2* and *Robo1* in a dose-dependent and time-dependent manner.

PGF $_{2\alpha}$ specifically increases *Slit2/Robo1* expression through PKC-dependent ERK1/2 and P38 MAPK signaling pathways

It has been reported that PGF $_{2\alpha}$ binds with its G-protein-coupled receptor and activates PKC and ERK1/2, which subsequently enhance related gene transcriptions in bovine luteal cells (Chen *et al.* 2001). In order to identify the signaling pathways that PGF $_{2\alpha}$ uses to affect the expression of *Slit2/Robo1* in mouse luteal cells, cultured luteal cells were separately pretreated with H89 (a PKA inhibitor), CH (a PKC inhibitor), PD98059 (an ERK1/2 inhibitor), SB203580 (a P38 inhibitor), and SP600125 (a JNK inhibitor) for 1 h, followed by incubation with 1 μM PGF $_{2\alpha}$ for 6 h. *Slit2* and *Robo1* mRNA levels were detected using real-time PCR. The results showed that CH, PD98059, and SB203580 blocked the enhancing effect of PGF $_{2\alpha}$ on the expression of *Slit2* and *Robo1*, whereas H89 and SP600125 did not modify *Slit2* and *Robo1* mRNA levels induced by PGF $_{2\alpha}$ (Fig. 4). These results indicate that PKC-dependent ERK1/2 and P38 MAPK signaling pathways are involved in the PGF $_{2\alpha}$ -induced *Slit2/Robo1* expression in mouse luteal cells.

PGF $_{2\alpha}$ increases *Slit2/Robo1* expression in the mouse CL

On D6 after hCG injection, the mice were i.p. injected with 10 μg cloprostenol, a synthetic analog of PGF $_{2\alpha}$. CL

Figure 1

Expression of the *Slit* and *Robo* family members in mouse ovary.

(A) mRNA levels of the *Slit* and *Robo* family members in the mid-CL of mice, as measured by real-time PCR. (B) Real-time PCR analysis of *Slit2* and *Robo1* mRNA levels in the staged mouse CL. Data in (A) and (B) are all means $\pm$ s.e.m. of three independent experiments done in triplicate and normalized to their respective control (* P <0.05 and ** P <0.01, by ANOVA). (C) Dual labeling of SLIT2 and ROBO1 was carried out in the

late-luteal-phase ovaries of mice. Green fluorescence indicates the localization of SLIT2. ROBO1-positive cells in the same field of the same slide were stained red fluorescence and the sections were counterstained with DAPI. NC, negative control. Arrows indicate representative double-stained cell, and all the scale bars represent 50 μ m. Three mice were examined in this experiment; a representative result is shown. F, follicle; and S, stroma.

tissues were collected at 0 h (control), 4, 12, and 18 h after cloprostenol injection. The mRNA levels of *Bax*, *Bcl2*, caspase9, caspase3, and *Slit2/Robo1* were detected using real-time PCR. The results showed that *Bax*, *Bax/Bcl2*, caspase9, and caspase3 mRNA levels were upregulated at 12 h (Fig. 5B and C, P <0.01), while *Slit2* and *Robo1* mRNA levels were significantly increased at 4 h (P <0.05), reaching a maximum at 12 h (P <0.05 and P <0.01 respectively), followed by a significant decline at 18 h (Fig. 5D and E). The protein levels of SLIT2 and ROBO1 were increased at 12 and 18 h (Fig. 5F and G, P <0.05 and P <0.01). These data confirm the *in vitro* results and demonstrate that

PGF $_{2\alpha}$ enhances the expression of *Slit2/Robo1* and enhances cell apoptosis in the CL *in vivo*.

***Slit/Robo* mediates the effects of PGF $_{2\alpha}$ on the apoptosis of luteal cells**

In order to determine whether the regulating effects of PGF $_{2\alpha}$ on CL regression are mediated by the *Slit/Robo* interaction, cultured luteal cells were treated either with or without PGF $_{2\alpha}$ in the presence or absence of ROBO1/Fc chimera for 24 h. Cell apoptosis was measured using an *in situ* apoptosis analysis kit. We detected apoptosis in

Figure 2

Effect of the *Slit/Robo* interaction on the apoptosis of mouse luteal cells. (A) Luteal cell apoptosis after 72 h treatment with ROBO1/Fc chimera or PBS/0.01% (w/v) BSA (control). The enlarged images of the boxed regions are shown on the right side of the merged pictures. Arrows indicate apoptotic nuclei. All the scale bars represent 50 μ m. (B) Percentages of

apoptotic cells accounting for the total luteal cells. (C) Caspase3 mRNA expression in the control and ROBO1/Fc chimera treatment groups was examined using real-time PCR. Results are means $\pm$ s.e.m. of three independent experiments done in triplicate and normalized to the control group (* $P < 0.05$, by *t*-test).

more than 5000 cells per treatment, and the results showed that the number of apoptotic cells after treatment with $\text{PGF}_{2\alpha}$ and the ROBO1/Fc chimera decreased by about 50% compared with that of cells treated with $\text{PGF}_{2\alpha}$ only (Fig. 6A and B). In addition, the expression of *Bax*, caspase9, and caspase3 was measured as shown in Fig. 6C. In the $\text{PGF}_{2\alpha}$ with ROBO1/Fc chimera-treated group, the expression of caspase9 and caspase3 was significantly decreased compared with that in the $\text{PGF}_{2\alpha}$ treatment group ($P < 0.05$). However, the expression level of *Bax* was not decreased. These data indicate that the blockade of the *Slit/Robo* interaction reduces the apoptosis of luteal cells induced by $\text{PGF}_{2\alpha}$.

Discussion

$\text{PGF}_{2\alpha}$ is the main luteolytic agent in many species, including mouse (Carambula *et al.* 2003). The *Slit/Robo* family members have been detected in the human ovary and found to be required for luteolysis (Dickinson *et al.* 2008). However, the functional relationship between

$\text{PGF}_{2\alpha}$ and *Slit/Robo* in the regulation of luteolysis has not been established. Our results showed that SLIT2 and ROBO1 are co-localized in luteal cells in the mouse ovary. Both *in vitro* and *in vivo* results first indicate that $\text{PGF}_{2\alpha}$ increases the expression of *Slit2/Robo1* both at gene and protein levels and the blockade of *Slit/Robo* signaling decreases the levels of apoptosis in luteal cells induced by $\text{PGF}_{2\alpha}$.

The *Slit/Robo* family comprises three ligands and four transmembrane receptors and their expressions have been detected in different tissues, such as neuronal tissue (Andrews *et al.* 2007), blood vessels (Jones *et al.* 2008, Koch *et al.* 2011), and the reproductive system (Dickinson *et al.* 2008, 2010, Duncan *et al.* 2010). The dominant cell types expressing SLIT2/ROBO1 in the human ovary are the granulosa luteal cells and theca luteal cells (Dickinson *et al.* 2008). In this study, the expression of the *Slit/Robo* family members in the mouse CL was detected and it was observed that *Slit2* and *Robo1* were co-expressed in luteal cells as indicated by IHC; these findings are in agreement with the results obtained in the

Figure 3

Upregulation of the expression of *Slit2/Robo1* in luteal cells by $\text{PGF}_{2\alpha}$ *in vitro*. (A) Expression levels of *Slit2* and *Robo1* were measured using real-time PCR after incubating the luteal cells with 0 (control), 0.01, 0.1, and 1 μM $\text{PGF}_{2\alpha}$ for 6 h. (B) mRNA levels of *Slit2* and *Robo1* at 0 (control), 3, 6, 12, and 24 h after incubating the luteal cells with 1 μM $\text{PGF}_{2\alpha}$. Results are means $\pm$ s.e.m. of three independent experiments conducted in triplicate and normalized to their respective control (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by ANOVA).

human ovary (Dickinson *et al.* 2008). The real-time PCR results showed that the abundance of *Slit2* was higher than that of *Slit1* and *Slit3*, and *Robo1* level was higher than *Robo2*, *Robo3*, and *Robo4* levels. These findings indicate that *Slit2* and *Robo1* have a function in the mouse CL.

Most functional studies on *Slit/Robo* have focused on cell migration, apoptosis, and tissue remodeling (Dallol *et al.* 2002, Hinck 2004). Since CL structural regression is characterized by cell apoptosis (Juengel *et al.* 1993, McCormack *et al.* 1998), we postulate that *Slit/Robo* is involved in the regulation of the apoptosis of mouse luteal cells. Our results demonstrate that *Slit2* and *Robo1* mRNA levels are significantly higher in the late luteal phase during which the CL sharply regresses. When *Slit/Robo* signaling is blocked, there is a sharp reduction in the number of apoptotic luteal cells and in the expression of genes that are closely associated with cell apoptosis. These

results demonstrate that the *Slit/Robo* interaction plays an important role in luteolysis in mice.

$\text{PGF}_{2\alpha}$ is important for luteolysis (Pharriss *et al.* 1972), and $\text{PGF}_{2\alpha}$ signaling involves its binding to a $\text{PGF}_{2\alpha}$ receptor and thus activating the PKC-dependent ERK1/2 pathway (Tai *et al.* 2001, Stocco *et al.* 2002). This study has proved that $\text{PGF}_{2\alpha}$ increases the expression of *Slit2/Robo1* in luteal cells *in vitro*. Furthermore, $\text{PGF}_{2\alpha}$ induces the expression of *Slit2/Robo1* in luteal cells through the PKC-dependent ERK1/2 and P38 MAPK signaling pathways, which subsequently enhance cell apoptosis in the CL. In addition, cloprostenol, a synthetic analog of $\text{PGF}_{2\alpha}$, also

Figure 4

Relative mRNA levels of *Slit2* (A) and *Robo1* (B) in cultured luteal cells that were treated with $\text{PGF}_{2\alpha}$ and several specific inhibitors. Inhibitors were added 1 h before the addition of $\text{PGF}_{2\alpha}$, and all the inhibitors were of a final concentration of 20 μM . After 6 h of exposure to $\text{PGF}_{2\alpha}$, the expression of *Slit2/Robo1* in each group was determined using real-time PCR. Results are all means $\pm$ s.e.m. of three independent experiments done in triplicate and normalized to the control group (* $P < 0.05$ and ** $P < 0.01$, by ANOVA; NS, no statistical significance).

Figure 5

Cloprostenol increases apoptosis-related genes and *Slit2/Robo1* expressions in CL *in vivo*. (A) Schematic representation of the experimental procedure for cloprostenol injection and CL collection in mice. (B) mRNA levels of *Bax*, *Bcl-2*, caspase9, and caspase3 in the CL following cloprostenol injection. (D) mRNA levels of *Slit2* and *Robo1* in the CL following cloprostenol injection. (F) Protein levels of SLIT2 and

ROBO1 in the CL at 0, 4, 12, and 18 h following cloprostenol injection. (C, E and G) Quantification of the gene/protein levels shown in (B), (D) and (F) respectively relative to those of GAPDH. GAPDH is an internal control. Results are means $\pm$ S.E.M. of three independent experiments done in triplicate and normalized to 0 h of injection (* $P < 0.05$ and ** $P < 0.01$, by ANOVA).

upregulates the expression of *Slit2/Robo1* in the CL *in vivo*. It is interesting that both *Slit2* and its *Robo1* receptor seem to be similarly regulated by cloprostenol at the same time, such a pattern of parallel ligand and receptor regulation indicates that the stimulation of this pathway has functional importance.

In order to assess whether the effect of $\text{PGF}_{2\alpha}$ on luteolysis depends on *Slit/Robo* signaling, we examined $\text{PGF}_{2\alpha}$ -induced luteal cell apoptosis with or without a *Slit/Robo* signaling inhibitor. Our results revealed that a *Slit/Robo* signaling inhibitor significantly decreases the levels of $\text{PGF}_{2\alpha}$ -induced luteal cell apoptosis. These findings demonstrate that $\text{PGF}_{2\alpha}$ and *Slit2/Robo1* are involved in the regulation of mouse luteolysis by their interactions. However, other death receptor-activating cytokines such

as tumor necrosis factor α (TNF α (TNF)) and FasL also mediate the effects of $\text{PGF}_{2\alpha}$ on luteolysis (Quirk *et al.* 2000, Pate & Landis Keyes 2001, Carambula *et al.* 2003).

Both the Robos and $\text{PGF}_{2\alpha}$ receptors are membrane receptors and their function is the promotion of cell apoptosis by the activation of apoptotic signaling cascades in the CL. It has been shown that $\text{PGF}_{2\alpha}$ interacts with its G-protein-coupled receptor to increase the ratio of *Bax* to *Bcl2*, which are closely associated with the elevation of the expression of caspase9 and caspase3 (Yadav *et al.* 2005). The mechanism by which *Slit/Robo* exerts this effect on apoptosis is unclear; however, the deleted in colorectal cancer (DCC) pathway may play a role in this progression. *Slit2* can bind to netrin-1 directly or can activate the interaction between Robo and DCC (Stein & Tessier-Lavigne 2001),

Figure 6

Reduction of PGF_{2α}-induced caspase-dependent apoptosis in mouse luteal cells by ROBO1/Fc chimera. (A) Mouse luteal cells treated with or without PGF_{2α} in the presence or absence of ROBO1/Fc chimera were probed using *in situ* apoptosis analysis. The enlarged images of the boxed regions are shown on the right side of the merged pictures. Arrows indicate apoptotic nuclei. All the scale bars represent 100 μ m. (B) Percentages of apoptotic

cells accounting for the total luteal cells. (C) mRNA levels of *Bax*, caspase9, and caspase3 in cultured luteal cells that were treated with or without PGF_{2α} in the presence or absence of ROBO1/Fc chimera for 24 h. Results are means $\pm$ S.E.M. of three independent experiments conducted in triplicate and normalized to that of the control group (* P < 0.05, by ANOVA; NS, no statistical significance).

which could, in principle, interfere with the netrin-1–DCC interaction. DCC can transmit pro-apoptotic signals in the absence of netrin-1 by activating caspase3 and caspase9 (Forcet *et al.* 2001). These findings suggest that caspase9 and caspase3 may be the mediating molecules between PGF_{2α} and *Slit/Robo* in their interactions in the progression of luteolysis. In support of this hypothesis, our results

demonstrate that the ROBO1/Fc chimera reduces cell apoptosis and caspase9 and caspase3 expression induced by PGF_{2α} in the luteal cells.

In conclusion, our results show that SLIT2 and ROBO1 are highly expressed and co-localized in the luteal cells of mice and they promote luteolysis by mediating the signaling pathway of PGF_{2α}-induced luteal cell apoptosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S C conceived and designed the experiments; X Z carried out the experiments; X Z, J L, and J L analyzed the data; H L and K G provided the reagents/materials/analysis tools; and S C and X Z wrote the manuscript.

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